FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)
198197US11PCT	09/647918

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/06525	14 April 1999	14 April 1998 (earliest)

TITLE OF INVENTION

SMALL MOLECULE ANTICANCER COMPOUNDS AND RELATED PRODUCTION PROCESS

APPLICANT(S) FOR DO/EO/US

Zhenhua YANG

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☐ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

Request for Consideration of Documents Cited in International Search Report
Notice of Priority
PCT/IB/304
PCT/IB/308

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/647918	PCT/US99/06525	198197US11PCT

20. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐ Search Report has been prepared by the EPO or JPO	$860.00	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482)	$690.00	
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	$710.00	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO	$1000.00	
☒ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	$100.00	
ENTER APPROPRIATE BASIC FEE AMOUNT =		$100.00
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		$130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	47 - 20 =	27	x $18.00	$486.00
Independent claims	10 - 3 =	7	x $80.00	$560.00
Multiple Dependent Claims (check if applicable).			☐	$0.00
TOTAL OF ABOVE CALCULATIONS			=	$1,276.00
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (check if applicable).			☐	$0.00
SUBTOTAL			=	$1,276.00
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).			+	$0.00
TOTAL NATIONAL FEE			=	$1,276.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).			☐	$0.00
TOTAL FEES ENCLOSED			=	$1,276.00
			Amount to be: refunded	$
			charged	$

☒ A check in the amount of $1,276.00 to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

22850

Surinder Sachar
Registration No. 34,423

Surinder Sachar
SIGNATURE

Harris A. Pitlick
NAME

38,779
REGISTRATION NUMBER

Oct 13 2000
DATE

TITLE OF THE INVENTION

~~SMALL MOLECULE ANTICANCER COMPOUNDS~~
AND RELATED PRODUCTION PROCESS

This application is a continuation in part of U.S. Application Serial No. 09/173,681 filed October 16, 1998, which application claims the benefit of U.S. Provisional Application 60/081,712, filed April 14, 1998.

BACKGROUND OF THE INVENTION

Field of the Invention:

The present invention relates to a group of compounds, i.e., specific branched-chain fatty acids, and pharmaceutically acceptable salts and derivatives thereof, with significant anticancer activities, and methods of treating cancer. The invention also relates to a process of producing fermentation products containing said specific branched-chain fatty acids, using specific bacteria strains, preferably in industrial facilities.

Description of the Background

Carcinoma is one of the most serious diseases threatening human's health and life. So far the predominant treatments to cancer patients are radiotherapy and chemotherapy. Both have certain toxicity or side effects on humans while suppressing cancer cell growth or killing cancer cells. Therefore extensive investigations have been carried out in order to find an effective anti-carcinogen with minimum side effects and toxicity.

In 1987, when the inventor cultured K562 leukemia cell lines in the laboratory, cells in a culture flask were found to have completely disappeared 48 hours after being contaminated by a kind of rod bacteria. Those rod bacteria were then intentionally harvested and purified, and incubated in soybean media with appropriate inorganic salts. It was found in later animal studies that the fermentation solution effectively inhibited tumor growth with no toxicity or side effects. In the decade since then, thousands of cancer patients, including advanced stage cancer patients, have been treated with the oral liquid developed from this fermentation solution. These include

leukemia, tongue cancer, colorectal cancer, breast cancer, prostate cancer, lung cancer, gastric cancer, hepatocarcinoma, melanocarcinoma, renal cancer, esophagus cancer and pancreas cancer patients. Most of them have responded to the oral liquid, such as by symptom improvement, tumor shrinkage or even complete disappearance. Many of these patients are still alive today. The cases included patients in China, Japan, Korea, the United States, and many other countries.

In order to discover the active components in the fermentation solution that play a key role in killing cancer cells, persistent investigations have been carried out for the last ten years. In this period many books and papers were published worldwide trying to explain the anticancer activity of this fermentation solution. Most of these reports suggested that some soybean isoflavones (e.g. genistein, daidzein and saponin) from the soybean media contributed to the anticancer activities of this fermentation solution. On the other hand, some clinical trials indicated that the anticancer activities of soybean isoflavones were not great enough to explain the anticancer effects of the fermentation solution. The inventor has isolated many compounds from the fermentation solution and revealed that the anticancer activities of the solution were largely contributed by 13-methyltetradecanoic acid and 12-methyltetradecanoic acid. Further investigations discovered that other members of the family of branched-chain fatty acids also had significant tumor-inhibition effects. So far there are no other reports in the literature regarding the anticancer activity of specific branched-chain fatty acids.

SUMMARY OF THE INVENTION

The present invention relates to a group of compounds, i.e., specific branched-chain fatty acids, and pharmaceutically acceptable salts and derivatives thereof, with significant anticancer activities, and methods of treating cancer using these compounds. Comprehensive biochemical and morphological tests have demonstrated that these activities are associated with induction of programmed cancer cell death (apoptosis). Very importantly, the specific branched-chain fatty acids do not kill normal cells. In animal studies, intraperitoneal injection of 13-methyltetradecanoic acid daily up to 800 mg/kg to mice did not reach the LD50 level (50% lethal dose). In human clinical studies, six volunteers received 0.6g – 1.8g 13-methyltetradecanoic acid daily for one month without any side effects.

The specific branched-chain fatty acids can be, but are not limited to, those obtained by synthesis, or by isolation from said fermentation products. Particularly, the present invention relates to the fermentation products containing these specific branched-chain fatty acids, which have the capability of inhibiting the growth of cancer cells without any toxic or side effects, and

the capability of antiaging and immune boosting as well. The present invention also relates to a process of producing fermentation products containing the specific branched-chain fatty acids, using specific bacteria strains, preferably in industrial facilities.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figures 1A and 1B show the morphological changes of K562 human leukemia cells undergoing apoptosis using transmission electron microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 4 hours.

Figures 2A and 2B show the morphological changes of SNU-423 human hepatocellular carcinoma cells undergoing apoptosis under a light microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 24 hours.

Figures 3A and 3B show the morphological changes of SNU-1 human gastric carcinoma cell lines stained with H&E under a light microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 8 hours.

Figures 4A and 4B show the morphological changes of DU-145 human prostate carcinoma cell lines stained with H&E under a light microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 8 hours.

Figures 5A and 5B show flow cytometric analysis of K562 human leukemia cells; A: untreated; B: treated with 13-methyltetradecanoic acid (30 μg/ml) for 24 hours.

Figures 6A, 6B and 6C show flow cytometric analysis of MCF-7 human breast adenocarcinoma cells; A: untreated; B: treated with 12-methyltetradecanoic acid (60 μg/ml) for 4 hours; C: treated with 12-methyltetradecanoic acid (60 μg/ml) for 24 hours.

Figures 7A and 7B show flow cytometric analysis of normal human peripheral blood lymphocytes (PBLs); A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 24 hours.

Figures 8A and 8B show detection of apoptotic SNU-1 cell lines added with TUNEL-(TdT-mediated dUTP nick end labeling) reaction mixture under a fluorescence microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 8 hours.

Figures 9A, 9B and 9C, show detection of apoptotic K-562 cell lines added with

peroxidase (POD) and substrate under a light microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 2 hours; C: treated with 13-methyltetradecanoic acid (60 μg/ml) for 4 hours.

Figures 10A and 10B show detection of apoptotic H1688 cell lines added with POD and substrate under a light microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 8 hours.

Figures 11A and 11B show detection of apoptotic DUI45 cell lines added with POD and substrate under a light microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 8 hours.

Figures 12A and 12B show normal human PBLs added with POD and substrate under light microscope; A: untreated; B: treated with 13-methyltetradecanoic acid (60 μg/ml) for 8 hours.

Figure 13 shows DNA fragmentation gel electrophoresis of K562 human leukemia cells undergoing apoptosis, which were treated with 13-methyltetradecanoic acid (60 μg/ml).

Figure 14 shows caspase target protein Lamin B cleavage in apoptotic SNU-423 human hepatocellular carcinoma cells treated with 13-methyltetradecanoic acid (60 μg/ml).

Figure 15 shows caspase target protein Lamin B cleavage in apoptotic K562 human leukemia cells treated with 13-methyltetradecanoic acid (60 μg/ml).

Figure 16 shows caspase target protein Rb hypophoshorylation and cleavage in apoptotic SNU-423 human hepatocellular carcinoma cells treated with 13-methyltetradecanoic acid (60 μg/ml).

Figure 17 shows caspase target protein Rb hypophoshorylation and cleavage in apoptotic K562 human leukemia cells treated with 13-methyltetradecanoic acid (60 μg/ml).

Figure 18 shows comparison of the tumors removed from the mice of two treated groups and control group of human prostate cancer DU145 nude mice model.

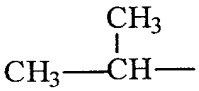
Figure 19 shows comparison of the tumors removed from the mice of treated group and control group of human hepatocellular carcinoma LCI-D35 orthotopic nude mice model.

DETAILED DESCRIPTION OF THE INVENTION

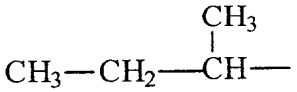
Definitions of the Specific Branched-chain Fatty Acids

The present invention relates to specific branched-chain saturated and unsaturated fatty acids, with significant anticancer activities, i.e., terminally methyl-branched iso- and anteiso-

fatty acids. The present invention also includes any and all derivatives of these fatty acids, so long as the terminally methyl-branched iso- or anteiso- fatty moiety remains. These fatty acids can be characterized by the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso- or anteiso- fatty group. By the term "terminally methyl-branched iso" and "terminally methyl-branched anteiso", it is intended that the end of the R_0 group farthest away from the COOH group have the following formulae, respectively:



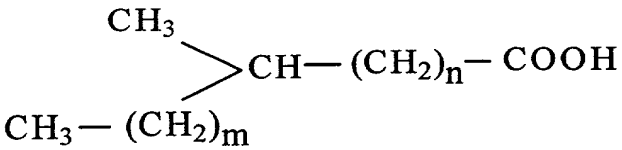
(iso fatty acid)



(anteiso fatty acid)

The portion of the fatty group R_0 other than the terminally-methyl branched iso- or anteiso- moiety, as described above, is not limited and may be saturated or unsaturated, linear or branched, for example.

An embodiment of the methyl-branched saturated fatty acids wherein the above portion of the fatty group R_0 other than the terminally-methyl branched iso- or anteiso- moiety is linear can be described by the formula (I):



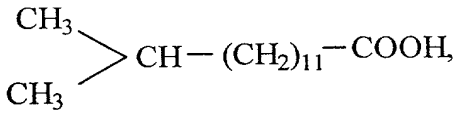
(I)

In the above formula (I), m is 0 or 1, and n is an integer. There is no lower or upper limit for n so long as the acid is a fatty acid. Thus, n + m may range as high as 96 or higher, with an upper limit of 46 being preferable. More preferably, n is 7-16.

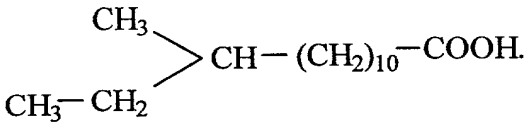
The methyl-branched unsaturated fatty acids have the above formula, except that n is at

least 2, and at least one CH_2-CH_2 group in $(CH_2)_n$ is replaced with a CH=CH group.

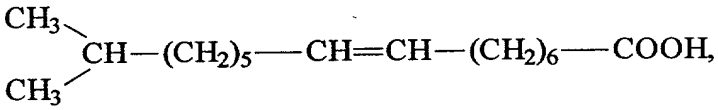
The terminally methyl-branched iso- fatty acids are the methyl-branched saturated fatty acids having x carbons and n = x – 4, m = 0 in the above formula, and known as "iso-Cx" in the present invention. For example, 13-methyltetradecanoic acid is expressed as "iso-C15" and has the formula



The terminally methyl-branched anteiso- fatty acids are the methyl-branched saturated fatty acids having x carbons and n = x – 5, m = 1 in the above formula, and known as "anteiso-Cx" in the present invention. For example, 12-methyltetradecanoic acid is expressed as "anteiso-C15" and has the formula



An example of a terminally methyl-branched unsaturated iso- fatty acid of the present invention is



otherwise known as iso-17:1 ω9c.

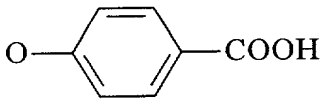
The present invention also includes pharmaceutically acceptable salts of said terminally methyl-branched iso- and anteiso- fatty acids, which are obtained by reaction with inorganic bases, such as sodium hydroxide, and have the ability to inhibit cancer cell growth. Such compounds include R_0COONa having not less than 12 carbons and R_0COOK having not less than 6 carbons, wherein R_0 is as defined above, Na is sodium, and K is potassium.

The present invention also includes pharmaceutically acceptable lipoproteins of said

terminally methyl-branched iso- and anteiso- fatty acids, which are obtained by conjugation with proteins, including polypeptides and oligopeptides, and have the ability to inhibit cancer cell growth. Such lipoproteins are well known in the art.

The present invention also includes all pharmaceutically acceptable derivatives other than lipoproteins, such as amides, esters, etc., of said terminally methyl-branched iso- and anteiso-fatty acids, which are obtained by reaction of the fatty acid with the corresponding amine, alcohol, etc. precursor, and have the ability to inhibit cancer cell growth. Such derivatives include, but are not limited to, those that have the formula R_0CO-A, where R_0 is as previously defined, and A represents one of the following groups:

1)



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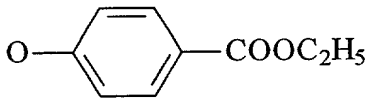

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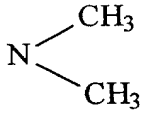

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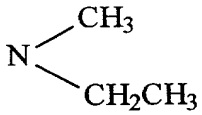

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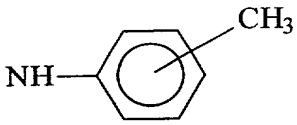

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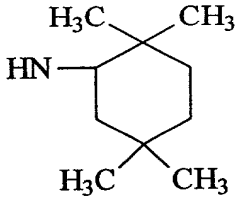

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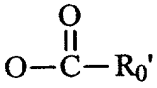

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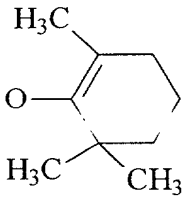

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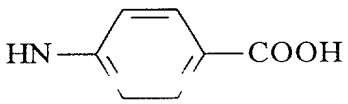

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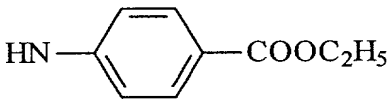

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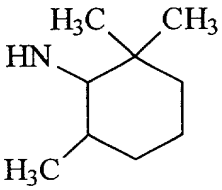

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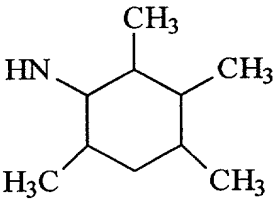
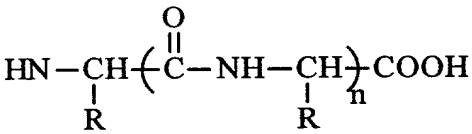


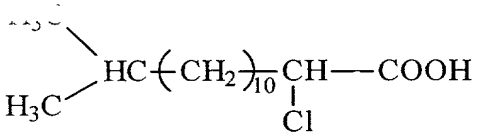
13)



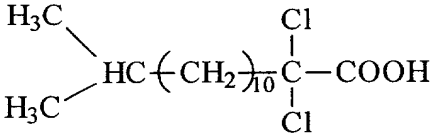
In the above formula 7, R_0' has the same definition as R_0 but may be the same or different. In the above formula 13, R is a side chain of an amino acid, and n is 0 or an integer.

The present invention also includes said terminally methyl-branched iso- and anteiso-fatty acids, wherein one or both hydrogens in a -CH_2 - group is substituted with a group X, such as Cl, I, Br, OH or NH_2, and have the ability to inhibit cancer cell growth. Examples of such

substituted fatty acids have the formula $R_0CHXCOOH$ or R_0CX_2COOH, and more than 8 carbon atoms, wherein R_0 is as defined above. Such compounds include:



and



The present invention also includes pharmaceutically acceptable salts, lipoproteins, and other derivatives of the above substituted fatty acids.

The terminally methyl-branched iso- and anteiso- fatty acids of the present invention can be obtained by, but not limited to, isolation from fermentation or incubation products using specific bacteria, or by chemical synthesis, or by extraction from natural materials.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

I. Demonstration of Anticancer Activity and Safety of Specific Branched-chain Fatty Acids

Example 1: Anticancer Activity *In Vitro*

Samples:

iso-C15, including extracted and synthesized.

The extracted iso-C15 was isolated by HPLC (High Performance Liquid Chromatography) from the fermented solution (fermented using the specific bacteria, *Stenotrophomonas maltophilia* Q-can, and media and production process in present invention).

The synthesized iso-C15 was purchased from Sigma Chemical Company (St. Louis,

MO.)

The other specific branched-chain fatty acids tested include:

10-methylundecanoic acid (iso-C12),

11-methyllauric acid (iso-C13),

12-methyltridecanoic acid (iso-C14),

11-methyltridecanoic acid (anteiso-C14),

12-methyltetradecanoic acid (anteiso-C15),

14-methylpentadecanoic acid (iso-C16),

13-methylpentadecanoic acid (anteiso-C16),

15-methylpalmitic acid (iso-C17),

16-methylheptadecanoic acid (iso-C18),

15-methylheptadecanoic acid (anteiso-C18),

17-methylstearic acid (iso-C19),

18-methylnonadecanoic acid (iso-C20).

All the samples above were purchased from Sigma Chemical Company.

Cell lines:

Human leukemia cell line K562 and human gastric cancer cell line SGC7901.

Methods:

MTT assay was performed to test the cytotoxicity. The K562 and SGC7901 cells were maintained in exponential growth in RPMI 1640 medium supplemented with 15% heat-inactivated newborn calf serum. The cells were plated at a density of 2 x 10^4 cells/100μl medium/well into 96-well plate with medium containing samples in five final concentrations (7.5, 15, 30, 60 and 90 μg/ml) for iso-C15 (either synthesized or extracted) and one final concentration (30 μg/ml) for the others. The media in control wells contained no samples. The cells were incubated at 37°C in a highly humidified incubator under 5% CO_2 atmosphere for 24 hours. The supernatant was removed by fast inversion of the plate. 20μl of 5mg/ml MTT solution were added into each well. Incubation was continued for 4 hours. DMSO 100μl/well was added and the plate was vibrated for 10 minutes. A_{570nm} was read at the Immunoreader BioTek EL311S.

The inhibition rate (%) = 1 - (mean A_{570nm} in test wells / mean A_{570nm} in control wells)

Results:

Table 1. Inhibitory rate (%) of synthesized iso-C15 * on cell growth

Cell line	90 μg/ml	60 μg/ml	30 μg/ml	15 μg/ml	7.5 μg/ml
K562	85.3	83.1	71.6	50.1	26.2
SGC7901	68.4	63.1	50.5	27.5	---

* the sample was dissolved with 10% ethanol.

Table 2. Inhibitory rate (%) of extracted iso-C15 * on cell growth

Cell line	90 μg/ml	60 μg/ml	30 μg/ml	15 μg/ml	7.5 μg/ml
K562	87.2	83.7	72.2	51.2	27.1
SGC7901	68.8	62.1	51.2	28.1	--

* the sample was dissolved with 10% ethanol.

Table 3. Inhibitory rate (%) of specific branched-chain fatty acids* on K562 cell growth

Sample	iso-C12	iso-C13	iso-C14	iso-C16	iso-C17	iso-C18
%	70.69	71.03	72.15	71.58	70.79	68.39
Sample	iso-C19	iso-C20	anteiso-C15	anteiso-C14	anteiso-C16	anteiso-C18
%	69.15	62.58	73.10	72.59	70.68	71.73

* the concentration of branched-chain fatty acids was 30μg/ml; the sample was dissolved with NaOH solution to adjust to pH 7.5.

Example 2: Determination of ID_{50}, ID_{75} and ID_{90}

Samples:

The extracted iso-C15 was isolated by HPLC from the fermented solution (fermented using the specific bacteria, *Stenotrophomonas maltophilia* Q-can, and process of the present invention, *infra*). The samples were prepared by dissolving them in NaOH solution (adjusted to pH7.5) and 0.5% Tween 80 (Sigma Chemical Company, St. Louis, MO).

Cell Lines:

All tumor cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VG) and were cultured as recommended by vendor. Human PBLs were separated from whole blood of healthy individuals by using Ficoll-Hypaque gradients. They were maintained in suspension in RPMI 1640 with 10% plasma from the same individuals. All cell cultures were incubated in a CO_2 atmosphere (5%) at 37°C.

Seven human tumor cell lines were studied. K-562 human leukemia and SNU-1 human gastric carcinoma cell lines were cultured in suspension in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS). MCF-7 human breast adenocarcinoma, DU- 145 human prostate carcinoma, SNU-423 human hepatocellular carcinoma, HCT 116 human colon carcinoma, and H1688 human small cell lung carcinoma cell lines were propagated as adherent cells in RPMI 1640 supplemented with 10% heat-inactivated FB S (for SNU-423 and H1688), or in McCoy's 5a medium with 10% heat-inactivated FBS (for HCT 116), or in minimum Eagle's medium with 10% heat-inactivated FBS (for MCF-7 and DU-145).

Methods:

All cells in adherent culture were initiated at 5 x 10^4 cells/well in 96-well microplates and treated immediately with iso-C15 at different concentrations (0, 1.5, 3.0, 6.0, 15.0, 30.0, and 60.0 μg/ml) diluted with medium. Both untreated and solvent (NaOH and Tween 80) treated cells served as controls. The treated cells were incubated for 48 hours at 37°C. After incubation, the supernatants were removed and the cells were trypsinized and collected prior to viability assessment by trypan blue dye exclusion.

PBLs, K-562 and SNU-1 cells in suspension culture were seeded in 96-well microplates at a density of 5 x 10^4 cells/well for K-562 and SNU-1, and 1 x 10^5 cells/well for PBLs. iso-C15 were diluted with medium to provide different concentrations (0, 1.5, 3.0, 6.0, 15.0, 30.0, and 60.0 μg/ml). Both untreated and solvent (NaOH and Tween 80) treated cells served as controls.

After incubation for 48 hours at 37°C, cells were collected directly from the wells for viability assessment.

The ID_{50}, ID_{75} and ID_{90} were determined in duplicate in every set of experiments, and each experiment was repeated three times under identical conditions. ID_{50}, ID_{75} and ID_{90} were defined as the concentration of iso-C15 required to kill 50, 75 or 90%, respectively, of cells (compared with that in untreated cells) and computed using CalcuSyn for Windows software (Biosoft, Cambridge UK) based on Median Effect method by Dr. T. C. Chou.

Results:

The cytotoxic activity of iso-C15 was quantified by determining ID_{50}, ID_{75} and ID_{90} (μg/ml or μM) in several human hematological and solid tumor cell lines. It is indicated from Table 4 that iso-C15 was active in all tumor cell lines studied. The strongest cytotoxic activities were for MCF-7 human breast adenocarcinoma and K-562 human leukemia. The activities were less for H1688 human small cell lung carcinoma and HCT 116 human colon carcinoma cell lines. In contrast, iso-C15 is not toxic against normal human peripheral blood lymphocytes at concentrations lethal to tumor cells.

Table 4. Cytotoxicity of iso-C15 on human tumor and normal cells *in vitro*

cell line	cell type	ID_{50}(μg/ml)	ID_{75}(μg/ml)	ID_{90}(μg/ml)
MCF-7	breast carcinoma	10.03±0.97	15.99±1.28	25.49±1.68
K-562	leukemia	11.45±1.82	22.27±4.60	43.57±6.71
DU145	prostate carcinoma	13.98±2.15	40.43±5.72	81.87±8.85
H1688	lung carcinoma	15.08±1.92	35.03±3.59	61.37±8.06
HCT-116	colon carcinoma	18.49±6.23	67.96±8.25	108.65±13.35
SNU-1	gastric carcinoma	20.77±2.47	47.43±4.95	80.49±10.03
SNU-423	hepatocarcinoma	24.26±3.98	70.46±9.36	120.77±15.82
PBL	normal human lymphocytes	>400		

Example 3: *In vitro* introduction of apoptosis in human tumor cell lines and molecular pathway

Reagents:

RPMI 1640, DMEM and McCoy culture medium, as well as Fetal and calf bovine serums were purchased from Life Technologies (Long Island, New York). Argarose for DNA gel electrophoresis was purchased from FMC, and Acrylamide for Western blot was from Bio-Rad. Antibodies against human c-myc, caspase 3, caspase 8, poly (ADP-ribose) polymerase (PARP), lamins, p53 and retinoblastoma (Rb) were from Oncogene. Chemicals used in buffers and other reagents were from Sigma (St. Louis, MO).

13-methyltetradecanoic acid (iso-C15) was chemically synthesized in the inventor's laboratory, as described in Example 5, *infra*, (purity of 99.8%) and 12-methyltetradecanoic acid (anteiso-C15) purchased from Sigma were prepared by dissolving in NaOH solution and then in 0.5% Tween 80 with pH7.5.

Cell Culture:

Human cancer cell lines DU-145 (prostate cancer), K562 (leukemia), HCT116 (colon cancer), H1688 (lung cancer), SUN423 (hepatocarcinoma), MCF7 (breast cancer), CRL-1687 (pancreatic cancer), and SUN-1 (gastric cancer) were obtained from American Type Culture Collection (ATCC). 30ml blood was collected from a health person and normal peripheral mononuclear cells were separated by Ficoll separation solution (Sigma). All cells were maintained in RPMI 1640, DMEM, or McCoy medium supplemented with 10% FCS, 100mg/ml streptomycin and 100u/ml penicillin. Normal human peripheral mononuclear cells, K562 and SUN-1 were suspended cells. After spinning at 1,500 RPM for 5 min, supernatants were discharged and cells were resuspended and expended in fresh medium. The other tumor cell lines were adherent cells and were dispersed with 0.05% trypsin / 0.01% EDTA (Irvine Scientific, CA) for expansion. Cells were seeded in T75 flasks at 2×10^6 cells/flask in culture medium supplemented with 10% fetal bovine serum and incubated overnight at 37°C with 5% CO_2. The adherent cells attached to the plate were striped with disposable cell scrapers (Fisher Scientific) after treated either with 1% iso-C15 , anteiso-C15 or control solution for 1, 2, 4, 8 and 24 hours and then combined with respective float cells. Cells were then prepared for flow cytometry analysis, *in situ* cell death detection, DNA fragmentation and Western blot assay followed the preparation methods for each assay. Cell pellets treated for 2 and 4hr with either

iso-C15 or control were also stored at -70°C for future studies of gene regulation.

Methods:

The apoptosis (programmed cell death) of cancer cells induced by specific branched-chain fatty acids was confirmed by: (a) morphology, visualizing morphological changes indicative of apoptosis; (b) flow cytometry, identifying the cells undergoing apoptosis and discriminating apoptosis from necrosis; (c) *in situ* cell death detection kit, POD, detecting apoptosis induced DNA strand breaks at single cell level; (d) gel electrophoresis assay, visualizing apoptotic DNA fragmentation.

The molecular mechanism of apoptosis induced by specific branched-chain fatty acids was studied using Western blot analysis.

A flow cytometer (FACScan) with Consort 30 software for gating analysis (Becton Dickinson, San Jose, CA) was used. The Apoptosis Detection kit (R&D Systems) was used to quantitatively determine the percentage of cells undergoing apoptosis by virtue of their ability to bind annexin V and exclude propidium iodide (PI). Cells were washed in cold PBS twice and resuspended in binding buffer. Fluorescent-labeled annexin V and PI were added to the cells. The cells undergoing apoptosis, expressing phosphotidyiserine on the outer leaflet of cell membranes, would bind annexin V. The cells in later stage of apoptosis or necrosis, with a compromised cell membrane, would allow PI to bind to the cellular DNA. The resulting cells were immediately analyzed by flow cytometer equipped with a single laser emitting excitation light at 488 nm. The annexin V and PI generated signals can be detected in signal detector FL1 and FL2, respectively. Three potential populations of cells can be presented in FL1/FL2 pattern: live cells would not stain with either fluorochrome (zone 3), necrotic and later apoptotic cells would stain with both fluorochromes (zone 2) while cells undergoing apoptosis would stain only with annexin V (zone 4).

In Situ Cell Death Detection Kit, POD (Mannheim Boehringer GmbH) was used to detect the individual apoptotic cells. Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments as well as single strand breaks in high molecular weight DNA. Those DNA strand breaks can be identified by labeling free 3'-OH termini with modified nucleotides in an enzymatic reaction. In this kit terminal deoxynucleotidyl transferase (TdT) is used to label free 3'-OH ends in genomic DNA with fluorescein-dUTP. The incorporated fluorescein is visualized under fluorescence microscope directly. The incorporated fluorescein can also bind to anti-fluorescein antibody POD and be detected by substrate reaction.

Stained cells can be analyzed under light microscope.

The gel electrophoresis assay was used for the detection of apoptosis-specific internucleosonal DNA degradation in these cells. Tumor cell pellets, treated with 1% iso-C15 and controls, were lysed in 1 ml hypotonic lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, and 0.2% Triton x-100). After centrifuged at 14,000 RPM for 20 min at 4°C, the supernatants were transferred to new tubes and treated with RNase and proteinase K respectively. Supernatants were extracted with phenol/chloroform twice, and fragmented DNA was precipitated in ethanol. Samples were electrophoresed in a 1.5% agarose gel in 1 × TAE buffer. The gel was stained with ethidium bromide and destained with distilled water. The fragmented DNA was then visualized under UV light.

For Western blot assay, each cell pellet collected from 1% iso-C15 or control treated cultures was lysed in 150 µl lysis buffer with 0.5% NP-40, 0.5% deoxycholic acid and 1mM PMSF. The cell lysates were mixed with equal volume 2 × Laemmli buffer and boiled for 5 min before loaded into gel wells. Proteins were resolved in an 8% SDS-PAGE gel and transferred to nitrocellulose filter membrane. The filters were blocked with PBS-T (PBS with 0.1% Tween 20) containing 5% nonfat dry milk (Bio-Rad, Richmond, CA) for 1 hr and then incubated for 1 hr with proper dilution of one primary antibody in PBS-T containing 2% nonfat dry milk. The filters then were washed in PBS-T 5 min for 6 times and incubated with a 1:8000 dilution of HRP secondary antibody in PBS-T with 2% nonfat dry milk for 1 hr. After 6 washes in PBS-T, immune complexes were visualized on film using the ECL nonradioactive detection system (Amersham, Arlington Heights, IL). After detected with one primary antibody, the filter was striped with 0.1 mM Tris pH 7.5 and 0.05 mM β-metacapenanol at 50°C for 30 min. The filters were washed in 300 ml PBS-T buffer for 10 min twice before blocking with PBS-T with 5% nonfat dry milk. The membranes were then reprobed with monoclonal mouse anti-human β-actin to determine the equal loading of protein for each well.

Results:

Morphological changes:

The apoptosis of cancer cells is morphologically characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, intact cell membrane and extensive formation of membrane blebs and apoptotic bodies.

Figure 1 shows the morphological changes of K562 leukemia cells undergoing apoptosis using transmission electron microscope. Comparing to the untreated intact cell (Figure 1A), the

cell treated with 13-methyltetradecanoic acid (60 μg/ml) for 4 hours (Figure 1B) exhibits typical apoptotic feature, chromatin condensed into dense masses against the nuclear membrane, membrane intact and cell shrinkage.

Figures 2–4 illustrate the morphological changes of cancer cells undergoing apoptosis in a light microscope. Cultured SNU-423 human hepatocellular carcinoma cells treated with anteiso-C15 (60 μg/ml) for 24 hours (Figure 2B) exhibited cell volume decrease due to shrinkage and bubbles inside the cell, compared to untreated control (Figure 2A). Cultured SNU-1 human gastric carcinoma cell lines were treated with anteiso-C15 (60 μg/ml) for 8 hours, and cellular morphology was evaluated in preparations stained with H&E (Figure 3B). Compared to untreated control (Figure 3A), chromatin condensation and cytoplasmic granularity were noted. Cultured DU-145 human prostate carcinoma cell lines were treated with iso-C15 (60 μg/ml) for 8 hours, and cellular morphology was evaluated in preparations stained with H&E dye (Figure 4B). Compared to untreated control (Figure 4A), membrane blebs were noted.

Flow Cytometry:

At least 10^4 cell events were analyzed. The FL1/FL2 pattern of untreated K562 human leukemia cells (Figure 5A) revealed the expected distribution of cells in zone 3. After treatment of K562 cells with iso-C15 (30 μg/ml) for 24 hours (Figure 5B), the majority of the cells were undergoing apoptosis (zone 4, Annexin V positive and PI negative). The kinetic behavior of anteiso-C15 in MCF-7 human breast adenocarcinoma cells was evidenced by Figure 6A, 6B and 6C, for treatment of MCF-7 cells with anteiso-C15 (60 μg/ml) for 0, 4 and 24 hours, respectively. After treatment of anteiso-C15 for 4 hours, many cells were undergoing apoptosis (zone 4, Figure 6B), while after 24 hours the majority of cells had already died (later stage of apoptosis, zone 2, Figure 6C). The flow cytometric analysis of untreated normal human PBLs (Figure 7A) and treated PBLs with iso-C15 (60 μg/ml) for 24 hours (Figure 7B) resulted in nearly identical FL1/FL2 patterns (zone 3, viable and not undergoing apoptosis), revealing no significant effects by iso-C15 on normal human lymphocytes.

In Situ Cell Death Detection:

Four human tumor cell lines, K-562 human leukemia, SNU-1 human gastric carcinoma cell lines, MCF-7 human breast adenocarcinoma and H1688 human small cell lung carcinoma cell lines, as well as Human PBLs were treated with iso-C15 (60 μg/ml).

SNU-1 cells treated with iso-C15 for 8 hours were added with TUNEL-reaction mixture and incubated 60 min at 37°C. After washing with PBS for three times, cell morphology was

analyzed directly under fluorescence microscopy. Several yellow fluorescent spots of apoptotic cells were noted in cells treated for 8 hours (Figure 8B), comparing to untreated ones (Figure 8A).

H1688, K-562 and DU145 human cancer cells and normal human PBLs were added with POD and incubated 30 min at 37°C, washed three times with PBS, then reacted with substrate AEC and incubated for 10 min at room temperature. The cells were analyzed under light microscope. Comparing K-562 leukemia cells untreated (Figure 9A) and treated for 2 and 4 hours (Figure 9B and 9C), it is found that some cells started apoptosis (stained red) 2 hours after treatment and the number of apoptotic cells increased with exposure time. The apoptotic H1688 cancer cells (stained red) were found after 8 hours of treatment (Figure 10B) comparing to untreated (Figure 10A). Some stained apoptotic DU145 cancer cells were shown 8 hours after treatment (Figure 11B) and no stained cells in untreated control (Figure 11A). In contrast, untreated and 8-hour treated PBLs were almost the same (Figure 12A and 12B), and few stained apoptotic cells were seen. It is evidenced that iso-C15 induces apoptosis of cancer cells but not normal human cells.

DNA Fragmentation Gel Electrophoresis:

DNA fragmentation electrophoresis is one of most common applied methods to illustrate the apoptotic changes in experimental cells. Results for K562 leukemia cell line treated with iso-C15 (60 μg/ml) were shown in Figure 13. The lane of control treated for 8 hours showed only DNA smear. The fragmented low molecular weight DNA bands were seen at 2 hour and were prominent at 8 hour treated. The appearance of an oligonucleosomal ladder in treated cells indicated the break of double-stranded DNA due to apoptosis induced by iso-C15.

Western Blot Analysis:

The Western blot analysis results (Figures 14–17) are used as examples to reveal the signal transduction pathway for specific branched-chain fatty acid to activate apoptosis of cancer cells.

The cleavages of Lamin B, a caspase target protein, in apoptotic SNU-423 human hepatocellular carcinoma cells and K562 human leukemia cells were shown in Figure 14 and 15, respectively. The cells were treated with 1% control solution and 1% iso-C15 for the length of time indicated. Cell lysates were separated by SDS-PAGE. Lamin B was detected by immunoblotting with a monoclonal antibody. The cleaved 45 kDa and 32 kDa products were shown in Figure 14, and the cleaved 45 kDa products in Figure 15. The cleavage of caspase

target protein Lamin B suggested the activation of the caspase cascade during apoptosis. The Western blot analysis of RB protein in SNU-423 and K562 cells were shown in Figure 16 and 17, respectively. The results showed that iso-C15 induced the change of hyperphosphorylated RB (pRB120/hyper) to hypophosphorylated form (pRB115/hypo), and also induced the cleavage of full length RB to pRB68 kDa fragment in Figures 16 and 17, and even smaller pRB48 kDa fragment in Figure 16.

Example 4: Anticancer Activity *In Vivo*

A. Determination of LD_{50}

Materials and methods:

13-methyltetradecanoic acid (iso-C15) purchased from Sigma (St. Louis, MO) was prepared by dissolving in NaOH solution and then in 0.35% Tween 80 with pH7.5.

ICR mice weighing 20.5 - 22.5 g of both sexes were treated with iso-C15 i.p. qd x 3 in test groups and with solvent of same dose as in a control group. The doses ranged from 10 to 800 mg/kg of iso-C15 and two mice were included in each dose group (10 mg/kg, 20 mg/kg, 40 mg/kg, 80 mg/kg, 160 mg/kg, and 800 mg/kg). The general condition of these mice were monitored daily for seven days.

Results:

No mice died after seven-day administration of iso-C15 of dose up to 800 mg/kg. It is shown that iso-C15 is basically not toxic to mice and 50% lethal dose (LD_{50}) was not determined.

B. Efficacy Evaluation of iso-C15 in Orthotopic Nude Mice Model of Human Prostate Carcinoma DU145

Material and Methods:

13-methyltetradecanoic acid (iso-C15) was chemically synthesized in the inventor's laboratory, as described in Example 5, *infra*, (purity of 99.8%) was prepared by dissolving in NaOH solution and then in 0.35% Tween 80 with pH7.5.

Total of 24 male athymic BALB/c nude mice between 4 and 5 weeks of age were bred and maintained in specific pathogen free condition.

Human prostate carcinoma DU145 tumor was implanted and maintained subcutaneously in the flank of athymic nude mice. Prior to orthotopic implantation, the tumor was harvested in log phase. The peripheral tumor tissue was collected and minced to small pieces of one cubic millimeter each.

The mice were anesthetized for surgical orthotopic implantation. A small incision was made along the midline of the lower abdomen. After proper exposure of the bladder and prostate, the capsule of the prostate was opened and three pieces of DU145 tumor fragments were inserted into the capsule. The capsule was then closed using 8-0 suture, and the abdomen was closed using a 6-0 surgical suture.

The mice bearing orthotopic DU145 were randomly divided into control and test groups of eight mice each at the second day after tumor implantation. The iso-C15 prepared above at doses 35 and 70 mg/kg and PBS were administered by gavage once a day in low dose and high dose test groups and control group, respectively, for 43 days.

All the mice were sacrificed by CO_2 inhalation at day-40 after the start of treatment. The weights of primary tumors and bodies were measured. Tissue samples of the primary tumors were processed through standard procedures of hematoxylin and eosin staining for microscopic examination.

The tumor inhibition rates (TIR) were determined by comparing the mean tumor weight of the test groups (T) with that of the control group (C) and expressed as a © - T)/C percentage, and were analyzed by Student's test for statistical significance.

Results:

Very promising antitumor efficacy was observed for iso-C15 at doses 35 mg/kg and 70 mg/kg in this nude mouse model of human prostate carcinoma DU145 with the tumor inhibition rates 54.8% ($p< 0.05$) and 84.6 % ($p< 0.01$) as shown in Table 5.

Table 5. Efficacy of iso-C15 on primary tumor and body weight in nude mouse model of human prostate carcinoma DU145

Group	Route	No. of mice	Mean tumor weight (mg)	TIR (%)	P
Control	Oral	8	1,090.75	--	--
Low dose, 35 mg/kg	Oral	8	493.25	54.8	0.042
High dose, 70 mg/kg	Oral	8	168.00	84.6	0.007

For comparison, all the primary tumors after removal from the nude mice are shown in Figure 18, where it is noted that the implanted tumor did not grow in four mice in the high dose treatment group. There are no signs of toxicity, as judged by the body weight curve and histology slides.

C. Efficacy Evaluation of iso-C15 in Orthotopic Nude Mice Model of Human Hepatocellular Carcinoma LCI-D35

Material and Methods:

13-methyltetradecanoic acid (iso-C15) was chemically synthesized in the inventor's laboratory, as described in Example 5, *infra*, (purity of 99.8%), was prepared by dissolving in NaOH solution and then in 0.35% Tween 80 with pH7.5.

Total of 16 male and female athymic BALB/c nude mice between 4 and 5 weeks of age were bred and maintained in specific pathogen free condition.

Human hepatocellular carcinoma LCI-D35 was originally obtained from the primary tumor of a 45-year-old female patient. The tumor was implanted and maintained subcutaneously in athymic nude mice. Prior to orthotopic implantation, the tumor was harvested in log phase. The peripheral tumor tissue was collected and minced to small pieces of one cubic millimeter each.

The mice were anesthetized for surgical orthotopic implantation. A small incision was made along the midline of the upper abdomen. The left lobe of the liver was exposed and a small incision was made on the liver surface. Two of the tumor fragments above were sutured into the incision using 8-0 suture. The abdomen was then closed using a 6-0 surgical suture.

The mice bearing orthotopic LCI-D35 were randomly divided into control and test groups of eight mice each at the second day after tumor implantation. The iso-C15 prepared above at dose 70 mg/kg and PBS were administered by gavage once a day in the test and control group, respectively, for 40 days.

All the mice were sacrificed by CO_2 inhalation at day-40 after the start of treatment. The weights of primary tumors and bodies were measured. Tissue samples of the primary tumors were processed through standard procedures of hematoxylin and eosin staining for microscopic examination.

The tumor inhibition rates (TIR) were determined by comparing the mean tumor weight of the test groups (T) with that of the control group (C) and expressed as a © - T)/C percentage, and were analyzed by Student's test for statistical significance.

Results:

Very promising antitumor efficacy was observed for iso-C15 at dose 70 mg/kg in this nude mouse model of human hepatocellular carcinoma LCI-D35 with a tumor inhibition rate 64.9 % ($p < 0.01$), as shown in Table 6.

Table 6. Efficacy of iso-C15 on primary tumor and body weight in nude mouse model of human hepatocellular carcinoma LCI-D35

group	dose	route	mice No. in. / fi.	body weight in. / fi.	tumor weight mean ± SD (g)	TIR (%)	p
PBS	------	oral	8 / 8	17.31/21.50	0.202 ± 0.117	------	
iso-C15	70mg/kg	oral	8 / 8	18.23/21.75	0.071 ± 0.052	64.9	0.0086

For comparison, all the primary tumors after removal from the nude mice are shown in Figure 19, where it is noted that the implanted tumor did not grow in two mice in the treatment group. There are no signs of toxicity, as judged by the body weight curve and histology slides.

D. Human Clinical Studies on the Safety of iso-C15

Material and Method:

Chemically synthesized iso-C15 of 99.8% purity were prepared in 0.20g capsules. Six healthy adult volunteers (4 male, 2 female) of average age 35.6 were divided into three groups, and orally received iso-C15 capsules for thirty days. Low dose group: one case, 0.6g daily; middle dose group: two cases, 1.2g daily; high dose group: three cases, 1.8g daily.

The examinations were carried out before, during and after experiment, including physical examinations, blood and urine routine examinations, function of heart, liver and kidney, X-ray radioscopy of lung and subjective symptom.

Results:

The effects of iso-C15 on blood routine and platelet shown in Table 7 indicated significant increase of white blood cells (WBC) and granulocyte (GRAN), while no significant changes of red blood cells (RBC), hemoglobin (HB) and platelet (PLT). No abnormality was observed from alanine aminotransferase (ALT) and blood urea nitrogen (BUN) for all subjects, as shown in Table 8. No abnormality was observed on heart and lung from electrocardiogram (EKG) and X-ray radioscopy, and no abnormality on urine routine as well.

C. Effects on chemotherapeutic toxic reaction

Table 19. Effects on toxic reaction of blood system

item	subgroup	case	Pre-treat (X±SD)	Post-treat (X±SD)	p
WBC($\times 10^9$)	test	30	4.74±1.21	5.45±0.86	<0.01
	control	30	5.29±0.85	4.45±0.80	
Neutrophil cell	test	30	3.20±0.82	3.66±0.69	<0.01
	control	30	3.72±0.58	3.09±0.45	
Hb (g/L)	test	30	94.63±18.00	96.89±16.08	<0.01
	control	30	103.67±13.24	99.20±11.63	
platelet($\times 10^9$/L)	test	30	140.30±4.88	160.03±4.36	<0.01
	control	30	157.33±3.52	145.53±5.33	

The blood routine and platelet quantity in the test subgroup dropped less than those in the control subgroup. This indicated that Q-can oral liquid can prevent the hemogram decrease caused by chemotherapy. Meanwhile, Q-can oral liquid was effective on the patients whose WBC and Hb were lower than normal before chemotherapy.

Table 20. Effects on hepatic function of the chemotherapy group

subgroup	case	SGPT (nmol/L, X+SD)		p
		pre-treat	post-treat	
test	89	460.06±25.34	330.11±245.01	<0.05
control	84	261.47±191.23	284.00±217.30	

Table 21. Effects on serum protein of chemotherapy

item	subgroup	case	pre-treat (g/L, X±SD)	post-treat (g/L, X±SD)	p
total protein	test	101	65.31±10.01	67.47±5.99	<0.01
	control	103	65.64±6.53	64.20±6.07	
albumin	test	107	38.78±5.65	39.13±5.26	<0.01
	control	102	39.44±4.74	38.18±5.24	

SGPT decreased and serum total protein increased in the test subgroup of combining

chemotherapy and Q-can oral liquid. The results showed that Q-can oral liquid could alleviate the damage of hepatic functions caused by chemotherapy and promote protein synthesis, thus protecting the liver.

Table 22. Effects on renal functions of the chemotherapy group

subgroup	case	Blood urea nitrogen (nmol/L)		case	Blood creatine (nmol/L)		p
		Pre-treat	post-treat		Pre-treat	Post-treat	
test	111	5.13±2.95	4.95±1.33	110	97.15±30.64	97.99±23.46	<0.01
control	100	4.26±1.03	5.04±1.42	90	89.28±22.13	107.08±41.27	

Blood urea nitrogen and creatine decreased in the test group, which indicated that Q-can oral liquid could alleviate the damage of renal function caused by chemotherapy.

In summary, compared with the chemotherapy only treatment of 131 cases of cancer patients, the results of combinational treatment with Q-can oral liquid showed markedly enhanced therapeutical effects with statistical significance. These effects included amelioration of the deficiency syndrome, improvement of the appetite, weakness, living quality and immune functions, mitigation of the degree of leucopenic action induced by chemotherapy, alleviation of the low leukocyte count and the hemoglobin concentration which decreased after treatment, and protection of the hepatic and the renal functions. In comparison with the radiotherapy only treatment, the amelioration of the deficiency syndrome and increase of the serum IgG level were found in cancer patients, who were treated by combination of radiotherapy with Q-can oral liquid. Q-can oral liquid had no toxic effects on the blood, heart, liver and kidney. Thus, Q-can oral liquid can be used as a supplementary therapeutic agent for cancer patients.

Example 16: Clinical Observation for 35 Cases of American Prostate Cancer Patients Treated by Q-can Oral Liquid

The effect of Q-can oral liquid on PSA levels was tested for 8-18 weeks (average 14 weeks) in two hospitals in the USA, where an integrative approach to treating prostate cancer was applied. Patients were not on radiotherapy, chemotherapy, or hormonal treatment during the recording period and followed a customized nutritional protocol. At a daily dosage of 250ml concentrated Q-can oral liquid (containing 300mg specific branched-chain fatty acids), assay of PSA level was made for all patients. The average drop in PSA level was noted. It is also found that drops in PSA level of the patients who had higher pre-treat PSA level was more significant than those of the patients who had lower pre-treat PSA level.

Table 23. The effects of Q-can oral liquid on PSA level (mg/ml)

Case Number	Pre-treat (mean±SD)	Post-treat (mean±SD)	p
35	10.2±10.72	7.45±6.06	<0.01

Example 17: Effects of iso-C15 on Psoriasis Skin Disease

13-methyltetradecanoic acid (iso-C15) was prepared by dissolving in NaOH solution and then in 0.8% Tween 80 with pH 7.5, with resulting concentration 10%. The iso-C15 cream was prepared with liposome technology.

Three psoriases patients topically applied iso-C15 cream on skin lesions three times per day for one month. The symptoms were obviously relieved (itching, flaking and red patches), and psoriases spots disappeared in one patient and 50% area reduced in the other two.

Example 18: Industrial Process for Making Fermentation Liquid

This example describes one method of industrial production of Q-can oral liquid in a more detailed manner.

Medium composition is: soybean 40kg (milling to milk and removing residue), K_2HPO_4 200mg, $CaCO_3$ 200g, yeast extract 160g, $MgSO_4$ 80g, NaCl 80g, Na_2MoO_4 10ppm. $ZnSO_4$ 10ppm, $CoCl_2$ 5ppm, $NaHNO_3$ 2ppm, soybean oil (as antifoam addition) 4kg, and add water to 400kg totally.

The above media is put into a seeding tank and lead steam 120°C for 30 minutes, then cooled to 30°C. Onto the seeding tank are inoculated 3kg liquid cultures, which were cultured on the incubator shaker at 30°C for 24 hours. Fermentation proceeds in the seeding tank for 24 hours, 30°C temperature, 200 rpm agitation speed, and 1:1 (v/v min) aeration rate. After confirming no infection under microscope, it is then transferred into a 10 ton production fermenter for 48 hours, where compared to that in the seeding tank before, the media is ten times in quantity and the same percentage of the composition, and the same parameters of temperature, agitation speed and aeration rate are used. When fermentation is finished and no infection is confirmed under microscope, the temperature is increased to 100°C to autoclave for 30 minutes. The cooled solution can be packaged and the packaged fermented solution is again autoclaved at 118°C for 45 minutes. This is a semi-finished product waiting for quality inspection and final package as the Q-can oral liquid product.

Every description in the above specification of a numerical range and of a genus is intended to inherently include a description of all possible values and subranges within the range, and all possible species and subgenuses within the genus, respectively.

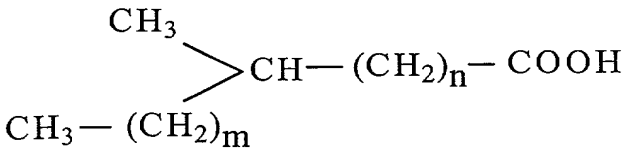
The disclosures of U.S. Application Serial No. 09/173,681 filed October 16, 1998, and of Provisional Patent Application No. 60/081,712, filed April 14, 1998, are hereby incorporated by reference.

Claims:

1. A method of treating cancer comprising administering to a cancer patient in need thereof an effective amount of at least one terminally methyl-branched iso- or anteiso-unsaturated fatty acid, or a pharmaceutically acceptable salt or derivative thereof, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso unsaturated fatty group.

2. The method of claim 1, wherein the portion of R_0 other than the terminally-methyl branched iso- or anteiso- moiety is linear or branched.

3. The method of claim 1, wherein the terminally methyl-branched iso- and anteiso-unsaturated fatty acids have the following formula (I):



(I)

where m is 0 or 1, and n is an integer between 7 and 16 inclusive, and at least one CH_2-CH_2 group in $(CH_2)_n$ is replaced with a CH=CH group.

4. The method of Claim 1, wherein the terminally methyl-branched iso- or anteiso-unsaturated fatty acid, salt or derivative thereof, is obtained by isolation from fermentation or incubation products using a bacteria strain containing said branched-chain fatty acid.

5. The method of Claim 4, wherein the bacteria strain is from a genus selected from the group consisting of *Stenotrophomonas, Xanthomonas, Flavobacterium, Capnocytophaga, Altermonas, Cytophage, Bacillus, Chryseobacterium, Empdobacter,*

Aurebacterium, Sphinggobacterium, Staphylococcus, Azotobacter and Pseudomonas.

6. The method of Claim 5, wherein the bacterial strain is *Stenotrophomonas maltophilia.*

7. The method of Claim 6, wherein said bacterial strain is assigned ATCC 202105.

8. The method of Claim 1, wherein R_0 represents a terminally methyl-branched iso-unsaturated fatty group, and the terminally methyl-branched iso- unsaturated fatty acid, salt or derivative thereof, is obtained by chemical synthesis.

9. The method of Claim 1, wherein the terminally methyl-branched iso- or anteiso-unsaturated fatty acid, salt or derivative thereof, is obtained by extraction from natural materials.

10. The method of Claim 1, wherein the terminally methyl-branched iso- or anteiso-unsaturated fatty acid is 15-methylhexadecenoic acid (iso 17:1 ω9c).

11. (Deleted)

12. The method of Claim 1, wherein the cancer treated is selected from the group consisting of leukemia, tongue cancer, colorectal cancer, breast cancer, prostate cancer, lung

cancer, gastric cancer, hepatocarcinoma, melanocarcinoma, renal cancer, esophagus cancer, pancreas cancer and skin cancers.

13. The method of Claim 1, wherein the terminally methyl-branched iso- or anteiso-unsaturated fatty acid, salt or derivative thereof, is administered as part of a fermentation product also containing a nutritive medium.

14. The method of Claim 13, wherein the nutritive medium comprises a soybean medium.

15. The method of Claim 14, wherein the soybean medium has the following formula:

Soybean	5-10 %
or soybean milk or bean cake (by soybean wt.)	5-15 %
Yeast extract	0.02-0.5 %
or yeast powder	0.02-0.5 %
$CaCO_3$	0.05-0.25 %
K_2HPO_4	0.02-0.10 %
$MgSO_4$	0.01-0.05 %
NaCl	0.01-0.04 %
Na_2MoO_4	5.0-30ppm
$ZnSO_4$	2.5-15ppm
$CoCl_2$	5.0-20ppm.

16. The method of Claim 15, wherein the fermentation product is obtained from a culture of *Stenotrophomonas maltophilia* assigned ATCC 202105.

17. The method of Claim 1, wherein the terminally methyl-branched iso- or anteiso-unsaturated fatty acid, salt or derivative thereof, is administered in the form of liquid, powder,

capsule, tablet, injection, or encapsulated liposome, or topically applied in the form of a cream, ointment, or lotion.

18. The method of Claim 1, wherein the terminally methyl-branched iso- or anteiso-unsaturated fatty acid is administered in the form of a pharmaceutically acceptable salt or derivative thereof.

19. A method of enhancing the treatment of cancer patients undergoing chemotherapy or radiotherapy comprising administering to a patient in need thereof an effective amount of at least one terminally methyl-branched iso- or anteiso-fatty acid, or a pharmaceutically acceptable salt or derivative thereof, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group.

20. The method of Claim 19, wherein the patient is undergoing chemotherapy and at least one of the following symptoms is treated: alleviation of the low leukocyte count and the hemoglobin concentration which is decreased after treatment, and protection of the hepatic and the renal functions.

21. The method of Claim 19, wherein the patient is undergoing radiotherapy, and at least one of the following symptoms is treated: amelioration of the deficiency syndrome and increase of the serum IgG level.

22. A method of treating a skin disease comprising administering to a subject in need thereof an effective amount of at least one terminally methyl-branched iso- or anteiso-fatty acid, or a pharmaceutically acceptable salt or derivative thereof, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group.

23. A method of making a terminally methyl-branched iso- or anteiso-fatty acid,

hemoglobin concentration which is decreased after treatment, and protection of the hepatic and the renal functions.

21. The method of Claim 19, wherein the patient is undergoing radiotherapy, and at least one of the following symptoms is treated: amelioration of the deficiency syndrome and increase of the serum IgG level.

22. A method of treating a skin disease comprising administering to a subject in need thereof an effective amount of at least one terminally methyl-branched iso- or anteiso-fatty acid, or a pharmaceutically acceptable salt or derivative thereof, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group.

23. A method of making a terminally methyl-branched iso- or anteiso-fatty acid, or a mixture of said fatty acids, which comprises culturing a bacteria strain containing said fatty acid(s) to form a fermentation solution containing said fatty acid(s), and then isolating said fatty acid(s), from the fermentation solution.

24. The method of claim 23, wherein the culture medium comprises a soybean medium.

25. The method of Claim 23, wherein the soybean medium has the following formula:

Soybean	5-10 %
or soybean milk or bean cake (by soybean wt.)	5-15 %
Yeast extract	0.02-0.5 %
or yeast powder	0.02-0.5 %
$CaCO_3$	0.05-0.25 %
K_2HPO_4	0.02-0.10 %
$MgSO_4$	0.01-0.05 %
NaCl	0.01-0.04 %
Na_2MoO_4	5.0-30ppm
$ZnSO_4$	2.5-15ppm
$CoC1_2$	5.0-20ppm.

26. The method of Claim 23, wherein the bacteria strain is from a genus selected from the group consisting of *Stenotrophomonas, Xanthomonas, Flavobacterium, Capnocytophaga, Altermonas, Cytophage, Bacillus, Chryseobacterium, Empdobacter, Aurebacterium, Sphinggobacterium, Staphylococcus, Azotobacter and Pseudomonas.*

27. The method of Claim 26, wherein the bacterial strain is *Stenotrophomonas maltophilia.*

28. The method of Claim 27, wherein said bacterial strain is assigned ATCC 202105.

29. A method of making a fermentation solution containing at least one terminally methyl-branched iso- or anteiso-fatty acid, which comprises culturing a bacteria strain containing said fatty acid in a nutritive medium to form a fermentation solution containing said fatty acid.

30. The method of Claim 29, wherein the nutritive medium comprising a soybean medium.

31. The method of Claim 30, wherein the soybean medium has the following formula:

Soybean	5-10 %
or soybean milk or bean cake (by soybean wt.)	5-15 %
Yeast extract	0.02-0.5 %
or yeast powder	0.02-0.5 %
$CaCO_3$	0.05-0.25 %
K_2HPO_4	0.02-0.10 %
$MgSO_4$	0.01-0.05 %
NaCl	0.01-0.04 %
Na_2MoO_4	5.0-30ppm
$ZnSO_4$	2.5-15ppm
$CoCl_2$	5.0-20ppm.

32. The method of Claim 29, wherein the bacteria strain is from a genus selected from

Aurebacterium, Sphinggobacterium, Staphylococcus, Azotobacter and Pseudomonas.

33. The method of Claim 32, wherein the bacterial strain is *Stenotrophomonas maltophilia.*

34. The method of Claim 33, wherein said bacterial strain is assigned ATCC 202105.

35. A product made by the method of Claim 29.

36. A product made by the method of Claim 30.

37. A product made by the method of Claim 31.

38. A product made by the method of Claim 32.

39. A product made by the method of Claim 33.

40. A product made by the method of Claim 34.

41. A composition comprising an effective amount for preventing cancer, or treating skin disease, or providing an antiaging effect, or providing immune boosting, of at least one terminally methyl-branched iso- or anteiso-fatty acid, or a pharmaceutically acceptable salt or derivative thereof, and a pharmaceutically acceptable carrier, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group.

42. The composition of Claim 41, wherein the composition is in the form of a liquid, powder, capsule, tablet, injection, or encapsulated with liposome, or topically applied

in the form of a cream, ointment, or lotion.

43. (Deleted)

44. (Deleted)

45. (Deleted)

46. The method of Claim 1, wherein the effective amount is an amount effective to induce apoptosis of cancer cells.

47. A method of immune boosting comprising administering to a subject in need thereof an effective amount of at least terminally methyl-branched iso- or anteiso-fatty acid, or a pharmaceutically acceptable salt or derivative thereof, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group.

48. A method of prolonging aging comprising administering to a subject in need

thereof an antiaging effective amount of at least one terminally methyl-branched iso- or anteiso-fatty acid, or a pharmaceutically acceptable salt or derivative thereof, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group.

49. A method of preventing cancer comprising administering to a subject in need thereof an effective amount of at least one terminally methyl-branched iso- or anteiso-fatty acid, or a pharmaceutically acceptable salt or derivative thereof, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group.

50. The method of Claim 49, where the cancer is skin cancer or mammary cancer.

51. (Deleted)

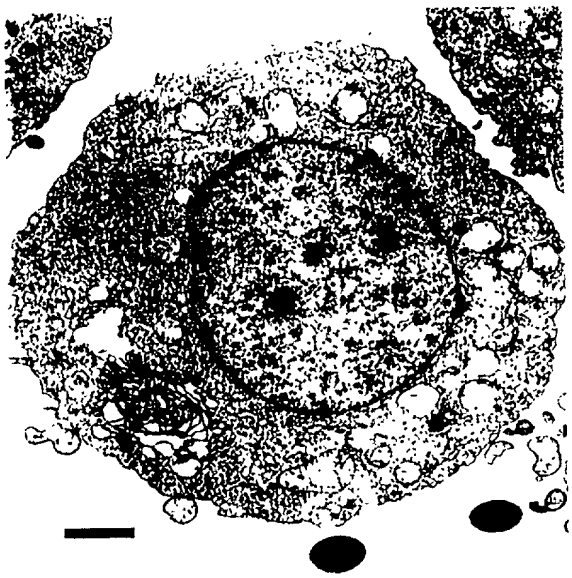
52. A terminally methyl-branched iso- or anteiso-fatty acid derivative, wherein the fatty acid has the formula R_0COOH, wherein R_0 represents a terminally methyl-branched iso or anteiso fatty group, and wherein said fatty acid derivative has anticancer activity, selected from the following compounds:

(1) R_0CO-A, wherein A represents one of the following groups:

SMALL MOLECULE ANTICANCER COMPOUNDS AND RELATED PRODUCTION PROCESS

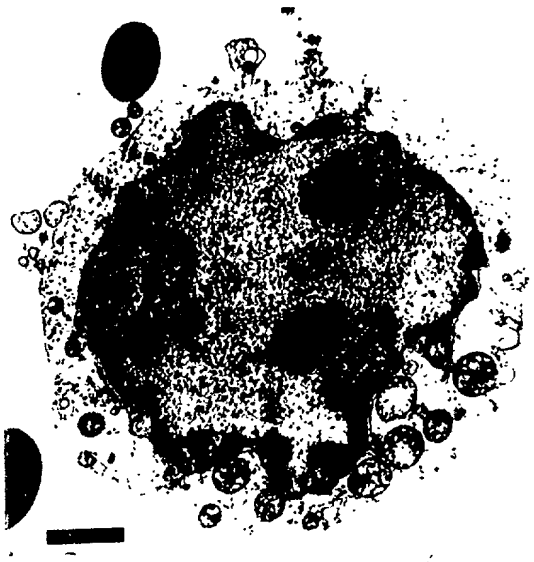
ABSTRACT OF THE DISCLOSURE

A group of specific branched-chain fatty acids, with significant anticancer effects on human and animals; methods of making using either chemical synthesis or biosynthesis methods; and methods of treating cancer.



SCALE BAR
= 2 μM

FIG.1A



SCALE BAR
= 2 μM

FIG.1B

FIG.2A FIG.2B

FIG.3A FIG.3B

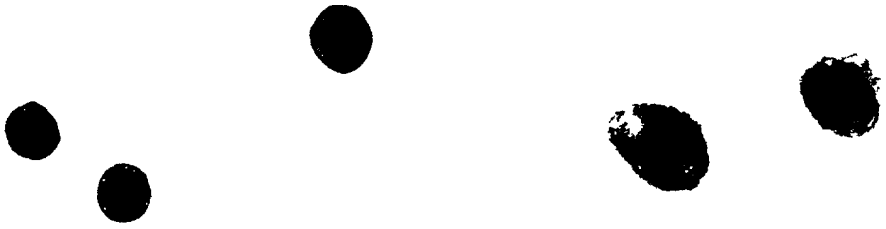


FIG.4A FIG.4B

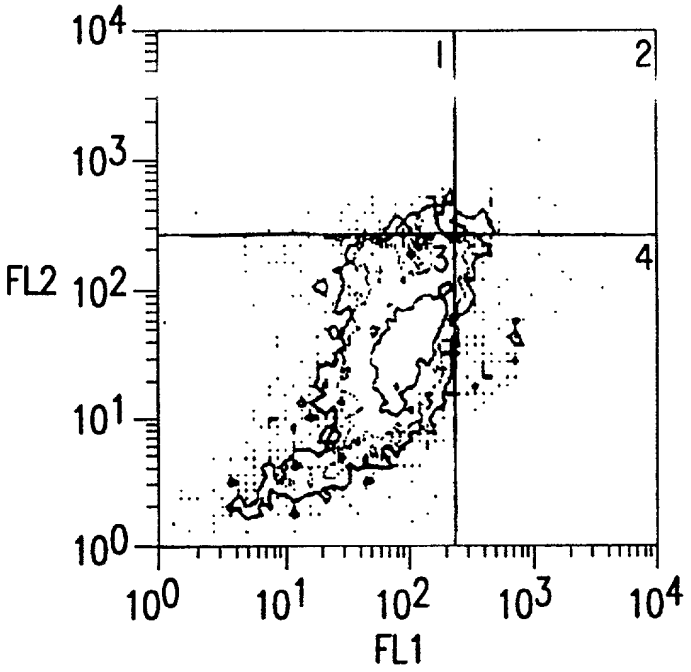


FIG.5A

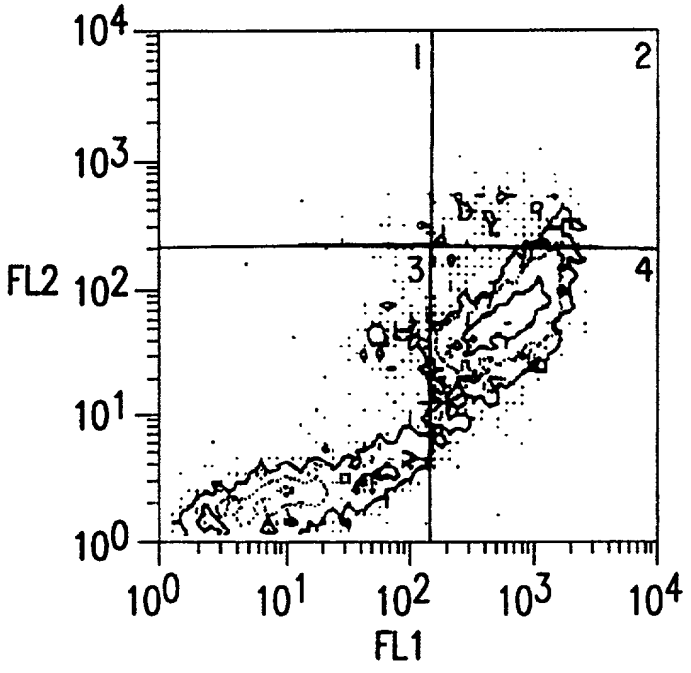


FIG.5B

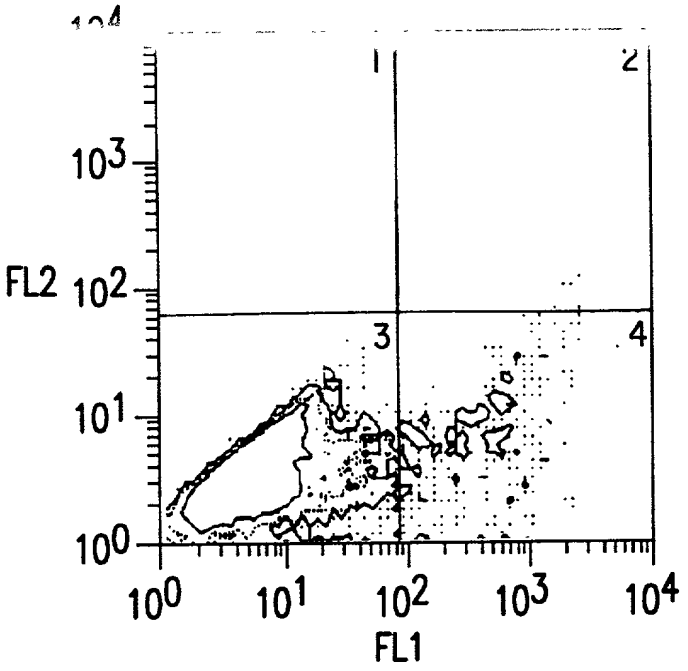

1
2
3
4
FL2
FL1
10^0
10^1
10^2
10^3
10^4

FIG.6A

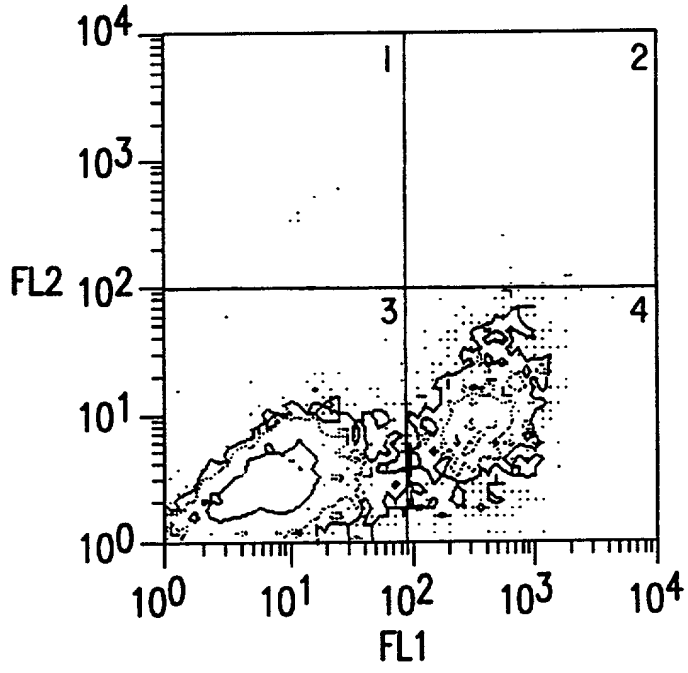

1
2
3
4
FL2
FL1
10^0
10^1
10^2
10^3
10^4

FIG.6B

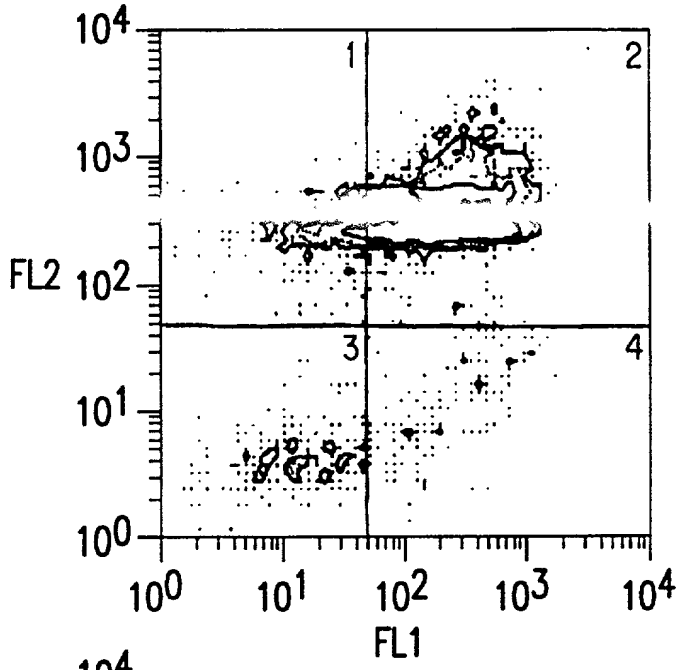


FIG.6C

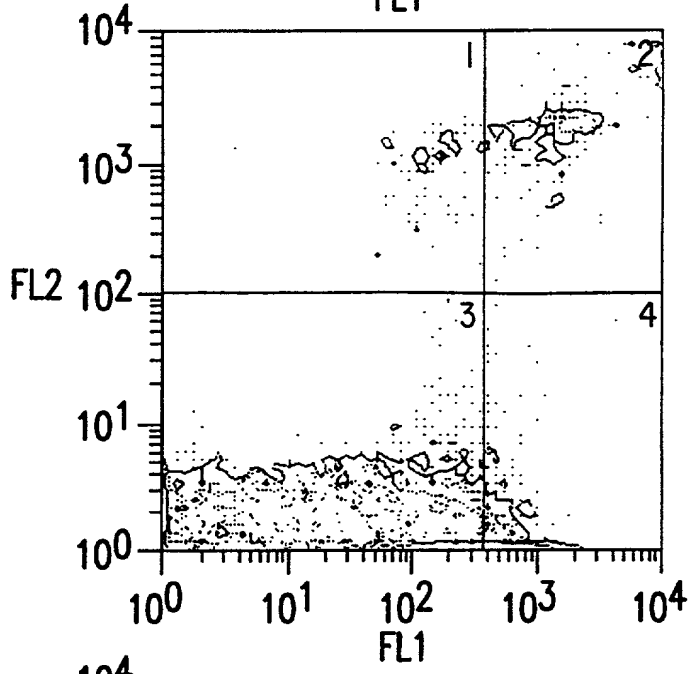


FIG.7A

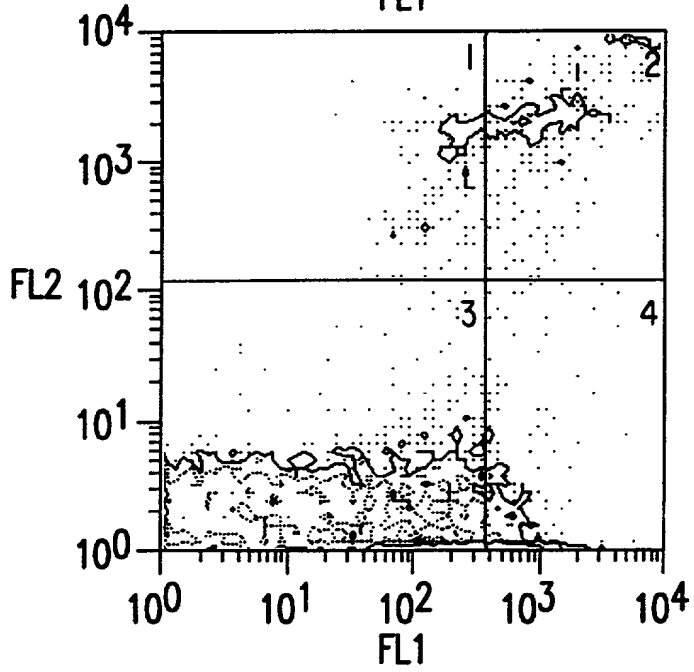


FIG.7B

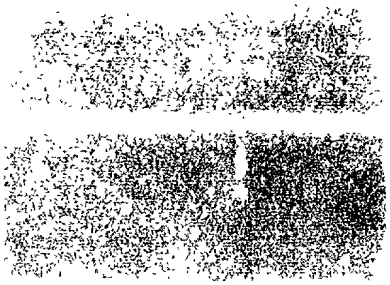


FIG.8A

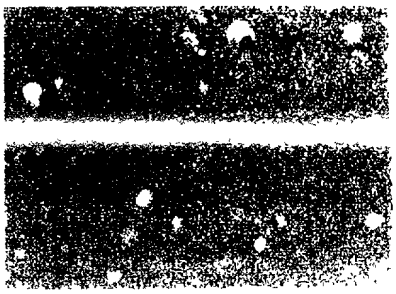


FIG.8B

FIG.9A

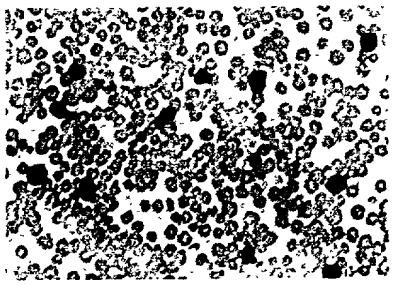


FIG.9B

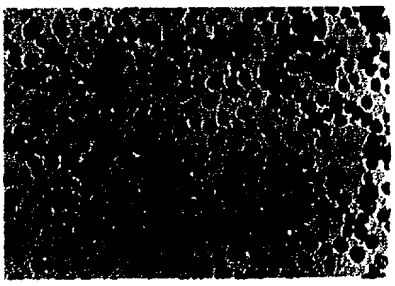


FIG.9C

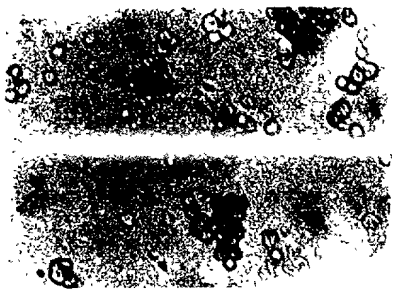


FIG.10A

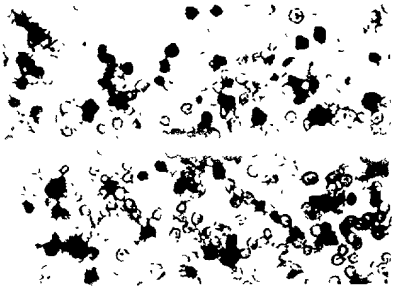


FIG.10B

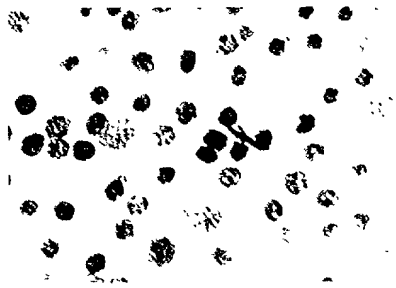


FIG.11A

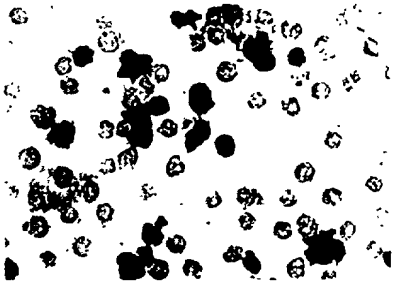


FIG.11B

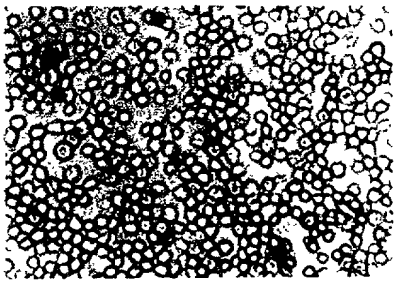


FIG.12A

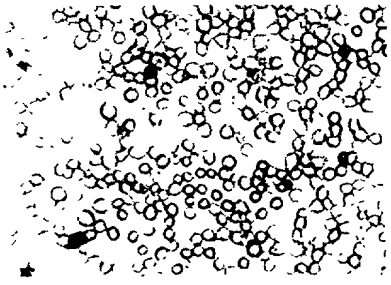


FIG.12B

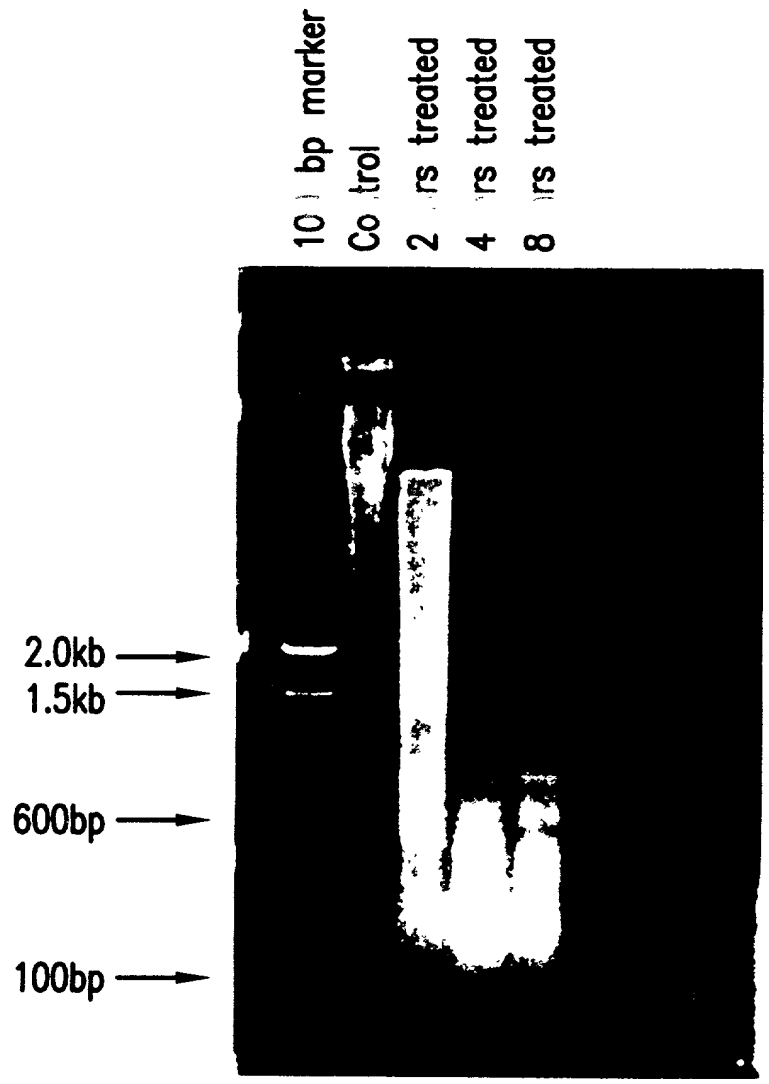


FIG.13

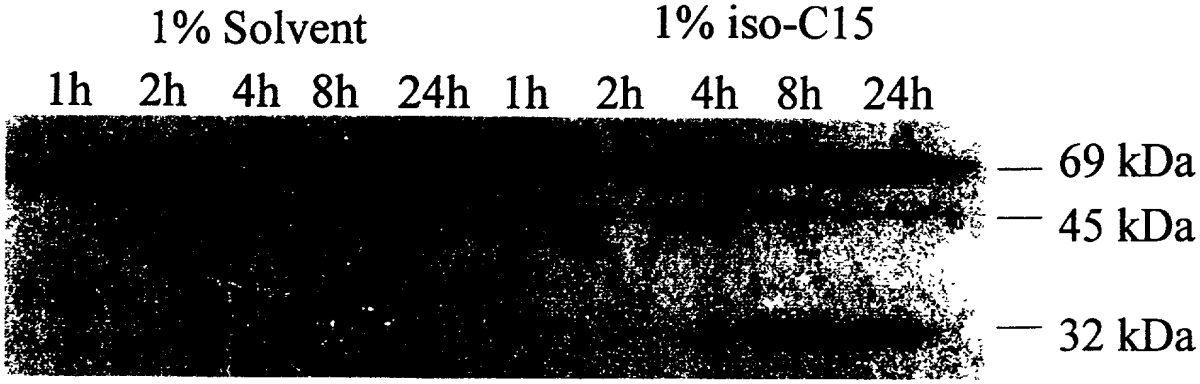


FIG.14

FIG.15

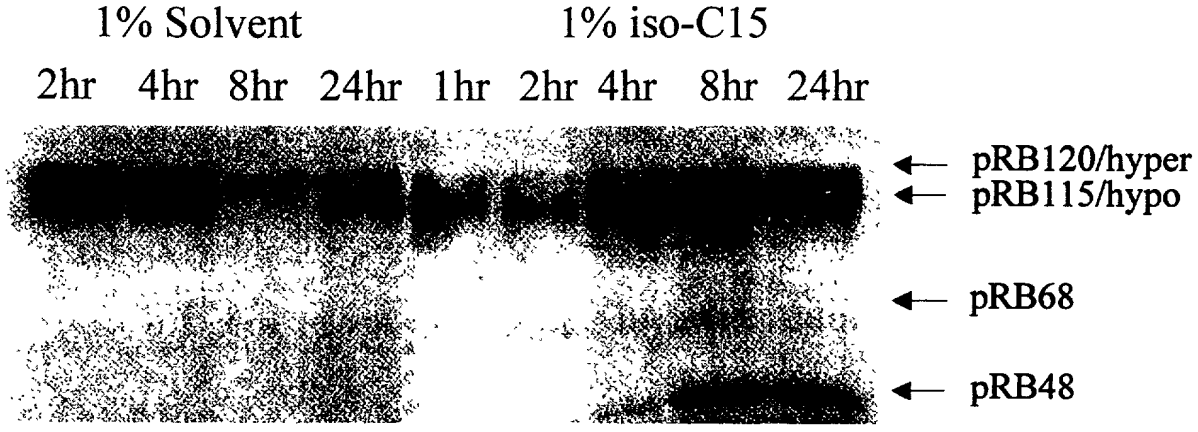


FIG.16

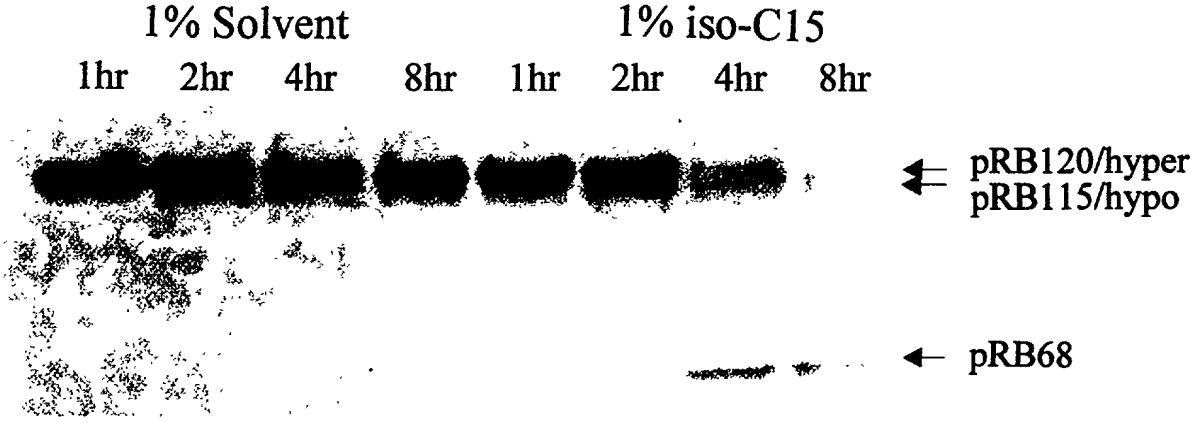


FIG.17

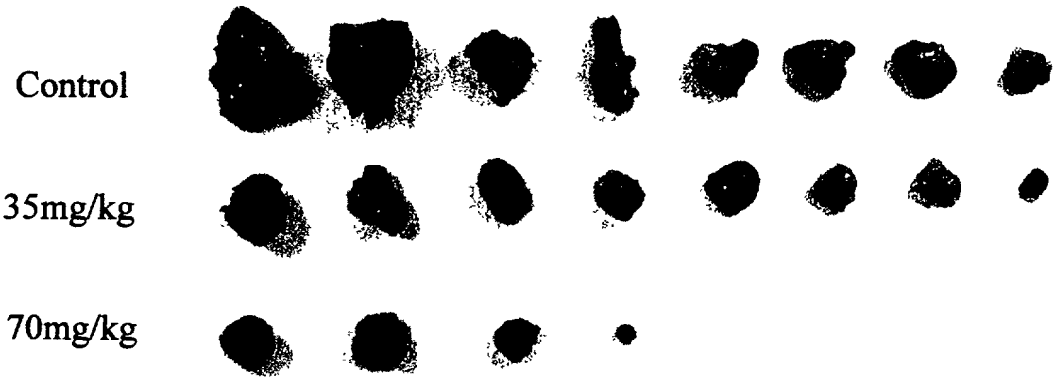


FIG.18

FIG.19

Declaration, Power Of Attorney and Petition

WE (I) the undersigned inventor(s), hereby declare(s) that:

My residence, post office address and citizenship are as stated below next to my name,

We (I) believe that we are (I am) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

SMALL MOLECULE ANTICANCER COMPOUNDS AND RELATED PRODUCTION PROCESS

the specification of which

☐ is attached hereto.

☒ was filed on October 13, 2000 as

Application Serial No. 09/647,918

and amended on _____.

☒ was filed as PCT international application

Number PCT/US99/06525

on April 14, 1999,

and was amended under PCT Article 19

on _____ (if applicable).

We (I) hereby state that we (I) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

We (I) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

We (I) hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed. Prior Foreign Application(s)

Application No.	Country	Day/Month/Year	Priority Claimed
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

We (I) hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

60/081,712	14 April 1998
(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

We (I) hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Application Serial No.	Filing Date	Status (pending, patented, abandoned)
PCT/US99/06525	14 April 1999	
09/173,681	16 October 1998	

And we (I) hereby appoint: Norman F. Oblon, Reg. No. 24,618; Marvin J. Spivak, Reg. No. 24,913; C. Irvin McClelland, Reg. No. 21,124; Gregory J. Maier, Reg. No. 25,599; Arthur I. Neustadt, Reg. No. 24,854; Richard D. Kelly, Reg. No. 27,757; James D. Hamilton, Reg. No. 28,421; Eckhard H. Kuesters, Reg. No. 28,870; Robert T. Pous, Reg. No. 29,099; Charles L. Gholz, Reg. No. 26,395; William E. Beaumont, Reg. No. 30,996; Jean-Paul Lavalleye, Reg. No. 31,451; Stephen G. Baxter, Reg. No. 32,884; Richard L. Treanor, Reg. No. 36,379; Steven P. Weihrouch, Reg. No. 32,829; John T. Goolkasian, Reg. No. 26,142; Richard L. Chinn, Reg. No. 34,305; Steven E. Lipman, Reg. No. 30,011; Carl E. Schlier, Reg. No. 34,426; James J. Kulbaski, Reg. No. 34,648; Richard A. Neifeld, Reg. No. 35,299; J. Derek Mason, Reg. No. 35,270; Surinder Sachar, Reg. No. 34,423; Jeffrey B. McIntyre, Reg. No. 36,867; William T. Enos, Reg. No. 33,128; Michael E. McCabe, Jr., Reg. No. 37,182; Bradley D. Lytle, Reg. No. 40,073; and Michael R. Casey, Reg. No. 40,294; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C., whose Post Office Address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202.

We (I) declare that all statements made herein of our (my) own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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28/02/2001
Date